

Genotyping Common Bean for the Potyvirus Resistance Alleles *I* and *bc-1²* with a Multiplex Real-Time Polymerase Chain Reaction Assay

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ABSTRACT

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A multiplex real-time polymerase chain reaction (PCR) assay was developed to simultaneously genotype plants for the *I* and *bc-1²* alleles, which condition resistance in beans to *Bean common mosaic virus* and *Bean common mosaic necrosis virus*. A segregating F₂ population was derived from the cross between pinto bean breeding line P94207-189A (*bc-1 bc-1 I I*) and Olathe (*bc-1² bc-1² i i*). Real-time PCR assays were developed that were specific for each allele, and a multiplex PCR reaction could unambiguously assign F₂ plants to one of nine genotypes. Remnant F₁ plants were used as a comparative reference sample. PCR results

among this sample fit a normal distribution for both real-time PCR assays, and 99% probability distributions were determined for heterozygotes. F₂ plants were genotyped based on results relative to the probability distributions for heterozygotes. F₂ plants also were genotyped for the *I* and *bc-1²* alleles by performing F₃ family progeny tests for virus resistance. Agreement between the two methods was 100% (198/198) for the *bc-1²* allele, and 92.4% (183/198) for the *I* allele. Erroneous genotyping was due to recombination between the amplicon and the *I* allele. Real-time PCR assays provide a robust method for genotyping seedlings and, in some cases, may eliminate the need for progeny testing.

Additional keywords: protected *I* gene, quantitative PCR.

Bean common mosaic virus (BCMV) and *Bean common mosaic necrosis virus* (BCMNV) are responsible for significant global production losses in common bean (*Phaseolus vulgaris* L.) (6). These viruses often are seed transmitted at high frequency; therefore, disease control is especially important for subsistence cultivation systems and in areas of seed production (6). The only effective means for controlling disease caused by these viruses is through the use of resistant cultivars (12).

Considerable efforts have been made to develop molecular markers that can be used to accelerate progress in breeding for disease resistance in common bean. The *bc-1²* allele confers resistance to BCMV strains within pathogroups I, II, and V and pathogroup III strains of BCMNV (6). Miklas et al. (14) developed SBD5₁₃₀₀, a sequence-characterized amplified region (SCAR) DNA marker (18), which appears to be tightly linked with *bc-1²*, because no recombination has been observed between SBD5₁₃₀₀ and *bc-1²* (15,20).

Similarly, Melotto et al. (14) developed the SCAR marker SW13₆₉₀, which is linked to the *I* allele. The *I* allele conditions resistance to all strains of BCMV and BCMNV (12). However, a temperature-insensitive hypersensitive necrosis response to all BCMNV strains and a temperature-sensitive necrosis response to a few BCMV strains in pathogroups IV and V results in the death of infected plants (5,8). Plant death induced by hypersensitive necrosis is called top necrosis. An epistatic interaction between *bc-1²* and *I* prevents the hypersensitive response from moving systemically, resulting in restricted vein necrosis instead of top necrosis and plant death (12). The combined effect of both the *bc-*

1² and *I* alleles confers a “protected *I* gene” response sought by breeders because it effectively provides resistance to all strains of BCMV and BCMNV (12). Linkage analysis indicates that *bc-1²* and SBD5₁₃₀₀, which are located on linkage group B3 (15), segregate independently from *I* and SW13₆₉₀, which are located on linkage group B2 (14).

Self pollination of an F₁ plant that is heterozygous for both the *bc-1²* and *I* alleles (*bc-1² bc-1 I i*) produces an F₂ population which, when infected with the NL-3 strain of BCMNV (pathogroup VI), segregates for genotypes resulting in four phenotypes at the following frequencies: nine restricted vein necrosis (*bc-1² _ I _*), three top necrosis (*bc-1 bc-1 I _*), three mild mosaic (*bc-1² _ i i*), and one mosaic (*bc-1 bc-1 i i*) (11). Because both SBD5₁₃₀₀ and SW13₆₉₀ are dominant SCAR markers, they cannot be used to distinguish between homozygous or heterozygous genotypes within the restricted vein necrosis phenotypic class. Consequently, the SBD5₁₃₀₀ and SW13₆₉₀ markers are not effective for identifying plants that are fixed at both loci for the desired resistance alleles (*bc-1² bc-1² I I*). Exclusive reliance on the use of SBD5₁₃₀₀ and SW13₆₉₀ for marker-assisted selection generally results in the selection of plants that express restricted vein necrosis but also are heterozygous for at least one of the two loci. Presently, no co-dominant markers have been identified that are linked to either the *bc-1²* or *I* alleles.

To maximize the likelihood of identifying F₂ plants that are fixed for both *bc-1²* and *I*, it is necessary to self pollinate plants that amplify both SBD5₁₃₀₀ and SW13₆₉₀ and then conduct F₃ progeny tests for resistance to BCMV or BCMNV. Plants that produce 100% resistant (restricted vein necrosis) F₃ progeny are homozygous at both loci for the resistance alleles, whereas plants that produce progeny that express any of the other three phenotypes (top necrosis, mild mosaic, or mosaic) are heterozygous for at least one of the two loci (6). The need to perform progeny testing to identify homozygous plants delays progress in improving disease resistance, because the process of self pollination, seed maturation, and progeny testing requires at least 4 months to complete.

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Segregation ratios indicate that only one of nine plants possessing at least a single dominant allele at both loci ($bc\text{-}I^2_I_$) will be fixed at both loci for the desired dominant alleles. Consequently, on average, eight of nine plants identified through marker-assisted selection with SBD5₁₃₀₀ and SW13₆₉₀ will not have the desired genotype. To identify the randomly amplified polymorphic DNA (RAPD) markers (21) that were used to develop these SCAR markers, 480 random primers were screened in the case of SW13₆₉₀ (9), and over 600 primers were evaluated prior to developing SBD5₁₃₀₀ (15). Given the difficulties encountered in developing these SCAR markers, it would be highly desirable if SBD5₁₃₀₀ and SW13₆₉₀ could be used more efficiently for bean improvement. A method that could discriminate simultaneously between homozygous and heterozygous plants for both SBD5₁₃₀₀ and SW13₆₉₀ would result in more efficient use of these markers.

Real-time polymerase chain reaction (PCR) assays that can quantify fluorescent-labeled DNA fragments may provide a method for simultaneously genotyping bean plants for both the SBD5₁₃₀₀ and SW13₆₉₀ markers. Real-time detection of PCR products (amplicons) can be performed using an amplification reaction that utilizes a pair of terminal oligonucleotide primers and a fluorochrome-labeled oligonucleotide probe that is homologous to an internal DNA sequence of the amplicon. Real-time PCR assays use the 5' nuclease activity of *Taq* polymerase to generate fluorescence in proportion to the number of target amplicons produced (11). A cycle threshold (C_T) is calculated for each sample, which is defined as the initial cycle number at which an increase in fluorescence above a baseline can be detected (10). The sample C_T values are used to calculate DNA quantity based on C_T values determined for standards containing known amounts of DNA.

Recently, a real-time fluorescent PCR assay based on SBD5₁₃₀₀ was developed to distinguish between heterozygous and homozygous genotypes for the linked $bc\text{-}I^2$ allele in a segregating common bean population (20). Remnant F_1 plants were used as a comparative reference sample. PCR results among this sample fit a normal distribution, and 99 and 95% probability distributions for heterozygotes were determined. F_2 plants were genotyped based on their results relative to probability distributions for heterozygotes. F_2 plants also were genotyped for $bc\text{-}I^2$ by self-pollination and performing F_3 family progeny tests for resistance to BCMNV. Agreement between the two different methods for genotyping plants was 100% (59/59) when PCR genotyping was based on a 99% heterozygote probability distribution, and 98.3% (58/59) when based on a 95% heterozygote probability distribution (20).

The objectives of this study were to (i) develop a real-time fluorescent PCR assay for the SW13₆₉₀ marker linked to the I allele and (ii) develop a multiplex real-time PCR assay (16) that could be used to simultaneously genotype bean plants for both the $bc\text{-}I^2$ and I alleles. F_3 progeny testing for resistance to BCMNV was used to validate the accuracy of the multiplex real-time PCR assay for genotyping F_2 plants.

MATERIALS AND METHODS

PCR primer and probe design. The population consisted of 198 F_2 plants derived from a cross between the pinto bean breeding line P94207-189A ($bc\text{-}I\ bc\text{-}I\ /\ I\ I$) and the pinto bean cv. Olathe ($bc\text{-}I^2\ bc\text{-}I^2\ /\ i\ i$) (22).

DNA was extracted from the first trifoliate leaf of each plant using the Fast-DNA kit (BIO 101 Inc., Carlsbad, CA). DNA was quantified with a fluorometer (TD-700; Turner Designs Inc., Sunnyvale, CA), and diluted to 20 ng/ μ l for use in quantitative PCR reactions.

The 697-bp DNA sequence of SW13₆₉₀ (GenBank accession no. AY508120), linked to the I gene, was analyzed using Primer Express software (Applied Biosystems, Foster City, CA) to

identify optimal sequences for real-time PCR primers and probes. The nucleotide sequences of the forward and reverse primers and the fluorochrome-labeled probe used in this study are as follows: forward primer I359PF, 5'-d-GGTCGTGGCTAGCGTTCTACA-3'; reverse primer I436PR, 5'-d-ATAGTGAGGACGTTAGAATA-GGAGTCTTC-3'; and probe I382TC, 5'-d-CACAGGAACAAC-GTGTCATCCGCAA-3'. The 5' terminus of the probe (TaqMan; Applied Biosystems) was labeled with the fluorochrome 6-carboxyfluorescein (6-FAM) and the 3' terminus was labeled with the quencher dye tetra-methylcarboxyrhodamine (TAMRA). The primer set I359PF-I436PR amplified a 78-bp fragment.

The primer/probe set p43335F-p43T369C-p43424R (20), previously designed based on the sequence of SBD5₁₃₀₀ (GenBank accession no. AY508119), was used to genotype plants for $bc\text{-}I^2$. The nucleotide sequences of the forward primer, reverse primer, and fluorochrome-labeled probe were as follows: forward primer p43335F: 5'-d-TGTACTGTGCTACCACTGCTACATCTT-3'; reverse primer p43424R, 5'-d-CAGAGCTCAGAATTGCAGCAA-3'; and probe p43T369C, 5'-ATGCTCCCTCACATTCATTAGTTTGCTGCATAT-3'. The 5' terminus of the probe (TaqMan; Applied Biosystems) was labeled with the fluorochrome VIC (Applied Biosystems) and the 3' terminus was labeled with the quencher dye TAMRA. The primer set p43335F-p43424R amplified a 90-bp fragment. All primers and probes were synthesized commercially (Applied Biosystems).

Multiplex TaqMan assays. Multiplex PCR for each plant sample was performed in 50- μ l reactions containing 100 ng of purified genomic DNA, 450 nM forward primer p43335F, 450 nM reverse primer p43424R, 250 nM TaqMan probe p43T369C, 450 nM forward primer I359PF, 450 nM reverse primer I436PR, 250 nM TaqMan probe p43T369C, and 25 μ l of 2 \times TaqMan Universal PCR Master Mix (Applied Biosystems). The GeneAmp 7000 Sequence Detection System (Applied Biosystems) was used for amplifications and detection of fluorescence. The thermocycling profile for all PCR reactions consisted of an initial cycle of 2 min at 50°C, then a single cycle of 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C.

Each set of PCR reactions included a nontemplate control reaction for which 100 ng of DNA was substituted with 5 μ l of double-distilled H₂O to confirm that the reagents were free of contaminating template DNA. For the primer/probe set I359PF-I382TC-I436PR (I allele), standard curves were generated for each set of PCR reactions using three replicate reactions each of 5, 25, 50, 100, and 200 ng of purified genomic DNA of the homozygous parent P94207-189A ($bc\text{-}I\ bc\text{-}I\ /\ I\ I$). For the primer/probe set p43335F-p43T369C-p43424R ($bc\text{-}I^2$ allele), standard curves were generated for each set of PCR reactions using three replicate reactions each of 5, 25, 50, 100, and 200 ng of purified genomic DNA of the homozygous parent Olathe ($bc\text{-}I^2\ bc\text{-}I^2\ /\ i\ i$).

Determination of plant genotype based on real-time PCR. Eleven remnant F_1 plants from the cross P94207-189A \times Olathe were included as comparative heterozygous ($bc\text{-}I^2\ bc\text{-}I\ /\ I\ i$) controls. Progeny testing, as described below, confirmed the heterozygous genotype of these 11 plants. For both SBD5₁₃₀₀ and SW13₆₉₀, a group mean (\bar{y}) and standard deviation (σ_y) was calculated for the comparative heterozygous F_1 plants based on the combined analysis of three PCR reactions for each plant. For both SBD5₁₃₀₀ and SW13₆₉₀, a Shapiro-Wilk test (19) was conducted to determine whether the real-time PCR results for the group of comparative heterozygous plants fit a normal distribution. Based on a normal distribution, a 99% probability distribution for heterozygotes was determined using the formula $\bar{y} \pm 2.58\sigma_y$ (17).

Real-time PCR results are expressed without units, because the PCR products amplified by the primer/probe sets specific for SW13₆₉₀ and SBD5₁₃₀₀ represent a very small fraction of the entire bean genome (78 and 90 bp, respectively). An equal amount of DNA (100 ng) was used for amplifications with all plant samples. Under ideal reaction conditions, in an absence of varia-

tion due to pipetting error or amplification efficiency, the primer/probe set I359PF-I382TC-I436PR should amplify twice as much of the 78-bp fragment of SW13₆₉₀ in *II* homozygous plants as in *Ii* heterozygotes. Similarly, the primer/probe set p43335F-p43T369C-p43424R should amplify twice as much of the 90-bp fragment of SBD5₁₃₀₀ in *bc-l² bc-l²* homozygotes as in *bc-l² bc-l* heterozygotes. However, because measurement variation generally is observed for any quantitative variable, discrimination between heterozygotes and homozygotes among the F₂ plants for the *I* and *bc-l²* alleles was based on comparisons with results for the reference sample of heterozygous F₁ plants (20).

The relative amounts of both *bc-l²* and *I* detected in each multiplex real-time PCR reaction were determined by plotting C_T values against the standard curves developed for each primer/probe set. The genotype of each F₂ plant for both *bc-l²* and *I* was determined based on the mean results of three multiplex real-time PCR reactions for each plant. Plants that were positioned within the 99% heterozygote probability distribution for SBD5₁₃₀₀ were classified as heterozygous for *bc-l²*. Plants that were positioned in the area to the right of the probability distribution were classified as homozygous for *bc-l²*. Plants for which no amplification was detected were considered homozygous recessive (*bc-l bc-l*). A χ^2 analysis was used to determine if results were not significantly different than the expected segregation ratio of 1 *bc-l² bc-l²*: 2 *bc-l² bc-l*: 1 *bc-l bc-l*. F₁ plants were genotyped similarly for *I*, based on the position of the mean calculated for each plant relative to the 99% heterozygote probability distribution determined with the real-time PCR assay specific for SW13₆₉₀. A χ^2 analysis was used to determine whether results were not significantly different than the expected segregation ratio of 1 *II*: 2 *Ii*: 1 *ii*. Finally, the independent assortment of alleles at both loci in the F₂ population was evaluated by conducting χ^2 analysis to determine if results were not significantly different than the expected segregation ratio of 1 *bc-l² bc-l² II*: 2 *bc-l² bc-l II*: 1 *bc-l bc-l II*: 2 *bc-l² bc-l² Ii*: 4 *bc-l² bc-l Ii*: 2 *bc-l bc-l Ii*: 1 *bc-l² bc-l² ii*: 2 *bc-l² bc-l ii*: 1 *bc-l bc-l ii*.

Determination of plant genotype based on progeny testing.

The 198 F₂ plants were grown in individual pots in the greenhouse to maturity. F₃ progeny were harvested individually from each F₂ plant. Twenty F₃ progeny from each F₂ plant were inoculated with the NL-3 strain of BCMNV. Inoculation procedures and greenhouse conditions followed those of Miklas et al. (15). F₂ plants were classified into one of nine possible genotype classes based on segregation within an F₃ family for disease reaction (restricted vein necrosis [VN], top necrosis [TN] mild mosaic [MM] or mosaic [M]) to the NL-3 strain as follows (7): *bc-l² bc-l² II* (F₃ = all VN), *bc-l² bc-l II* (F₃ = 3 VN:1 TN), *bc-l bc-l II* (F₃ = all TN), *bc-l² bc-l² Ii* (F₃ = 3 VN:1 MM), *bc-l² bc-l Ii* (F₃ = 9 VN: 3 TN: 3 MM: 1 M), *bc-l bc-l Ii* (F₃ = 3 TN:1 M), *bc-l² bc-l² ii* (F₃ = all MM), *bc-l² bc-l ii* (F₃ = 3 MM: 1 M), and *bc-l bc-l ii* (F₃ = all M). A χ^2 analysis was performed to determine if results were not significantly different than the expected segregation ratio of 1 *bc-l² bc-l² II*: 2 *bc-l² bc-l II*: 1 *bc-l bc-l II*: 2 *bc-l² bc-l² Ii*: 4 *bc-l² bc-l Ii*: 2 *bc-l bc-l Ii*: 1 *bc-l² bc-l² ii*: 2 *bc-l² bc-l ii*: 1 *bc-l bc-l ii*.

RESULTS

Primers/probe set sensitivity and specificity. The primer/probe sets could detect target DNA in assays using a range of 5 to 200 ng of template DNA. For primer/probe set I359PF-I382TC-I436PR, an $R^2 \geq 0.998$ was observed for the relationship between the log₁₀ of the initial DNA quantity and C_T value for all sets of reactions (data not shown). For primer/probe set p43335F-p43T369C-p43424R, an $R^2 \geq 0.997$ was observed for all sets of reactions. The primer/probe set I359PF-I382TC-I436PR did not amplify DNA from the parent Olathe (*bc-l² bc-l² ii*), confirming that that primer/probe set was specific for the SW13₆₉₀ marker

linked to the *I* allele (Fig. 1). Conversely, the primer/probe set p43335F-p43T369C-p43424R did not amplify DNA from the parent P94207-189A (*bc-l bc-l II*), confirming that that primer/probe set was specific for the SBD5₁₃₀₀ marker linked to the *bc-l²* allele (Fig. 1).

Determination of plant genotype for the *I* allele based on real-time PCR. Examples are presented of amplification plots obtained for reactions containing DNA from the parent P94207-189A (*bc-l bc-l II*), the parent Olathe (*bc-l² bc-l² ii*), and a heterozygous F₁ plant (*bc-l² bc-l Ii*) (Fig. 1A). The amplification plots obtained with primer/probe set I359PF-I382TC-I436PR, specific for SW13₆₉₀, shows that the homozygous dominant parent P94207-189A crossed the threshold ($\Delta R_n = 2.0$) before that of the heterozygous F₁ plant, indicating that more SW13₆₉₀ template is present in the homozygous dominant plant.

For assays conducted using primer-probe set I359PF-I382TC-I436PR, the mean of three replicate PCR reactions using 100 ng of genomic DNA per reaction was 102.61 for the homozygous parent P94207-189A (*bc-l bc-l II*). The Shapiro-Wilk test for data from three replicate PCR reactions for each F₁ plant indicated that the data fit a normal distribution (Table 1). The range of means among the reference sample of 11 F₁ heterozygote plants and the group mean and standard deviation for F₁ plants also are presented in Table 1. The 99% probability distribution for plants that were heterozygous for *I*, based on the data from the F₁ plants, was 38.13 to 61.48.

A frequency histogram for the distribution of real-time PCR results of the 198 F₂ plants is presented in Figure 2A. In all, 94 F₂ plants were positioned within the heterozygote probability distribution, and were classified as heterozygous (*Ii*). The Shapiro-Wilk test for data from three replicate PCR reactions for each *Ii* heterozygous F₁ plant indicated that the data fit a normal distribution (Table 1). In all, 41 F₂ plants were positioned in the area to the right of the heterozygote probability distribution and were classified as having a homozygous dominant genotype (*II*). The Shapiro-Wilk test for data obtained from three replicate PCR reactions for each *II* homozygous F₂ plant indicated that the data fit a normal distribution (Table 1). The range of means among the heterozygous and homozygous F₂ plants and the group mean and standard deviation for each genotypic class also are presented in Table 1.

Sixty-three F₂ plants were identified for which no DNA was amplified in any of three replicate PCR reactions, and these plants were classified as having a homozygous recessive genotype (*ii*). Classification of plant genotype based on the results of real-time PCR indicated that the 198 F₂ plants segregated as follows for the *I* allele: 41 *II*: 94 *Ii*: 63 *ii*. This did not significantly deviate from the expected 1:2:1 segregation ratio ($\chi^2 = 5.39$; df = 2; 0.05 < P < 0.10).

Determination of plant genotype for the *bc-l²* allele based on real-time PCR. Amplification plots obtained with primer/probe set p43335F-p43T369C-p43424R, specific for SBD5₁₃₀₀, which is linked to the *bc-l²* allele, are presented in Figure 1B. The homozygous dominant parent Olathe (*bc-l² bc-l² ii*) crossed the threshold ($\Delta R_n = 0.2$) before that of the heterozygous F₁ plant (*bc-l² bc-l Ii*), indicating that more SBD5₁₃₀₀ template DNA is present in the homozygous plant.

For assays conducted using primer/probe set p43335F-p43T369C-p43424R, the mean of three replicate PCR reactions using 100 ng of genomic DNA template per reaction was 110.50 for the homozygous parent Olathe (*bc-l² bc-l² ii*). The Shapiro-Wilk test for the data obtained from three replicate PCR reactions for each F₁ plant indicated that the data fit a normal distribution (Table 1). The range of means among the reference sample of 11 F₁ heterozygote plants and the group mean and standard deviation for F₁ plants are presented in Table 1. The 99% probability distribution for plants that were heterozygous for *bc-l²*, based on the data from the F₁ plants, was 31.33 to 59.53.

A frequency histogram for the distribution of real-time PCR results of the 198 F₂ plants is presented in Figure 2B. In all, 104 F₂ plants were positioned within the heterozygote probability distribution, and were classified as heterozygous (*bc-l² bc-l*). The Shapiro-Wilk test for the data obtained from three replicate PCR reactions for each heterozygous F₂ plant indicated that the data fit a normal distribution (Table 1). In all, 44 F₂ plants were positioned in the area to the right of the heterozygote probability distribution, and were classified as having a homozygous dominant

genotype (*bc-l² bc-l²*). The Shapiro-Wilk test indicated that the data obtained from three replicate PCR reactions for each *bc-l²* homozygous F₂ plant fit a normal distribution (Table 1). The range of means among the heterozygous and homozygous F₂ plants and the group mean and standard deviation for each genotypic class are presented in Table 1.

In all, 50 F₂ plants were identified for which no DNA was amplified in any of three replicate PCR reactions, and these plants were classified as having a homozygous recessive genotype (*bc-l*

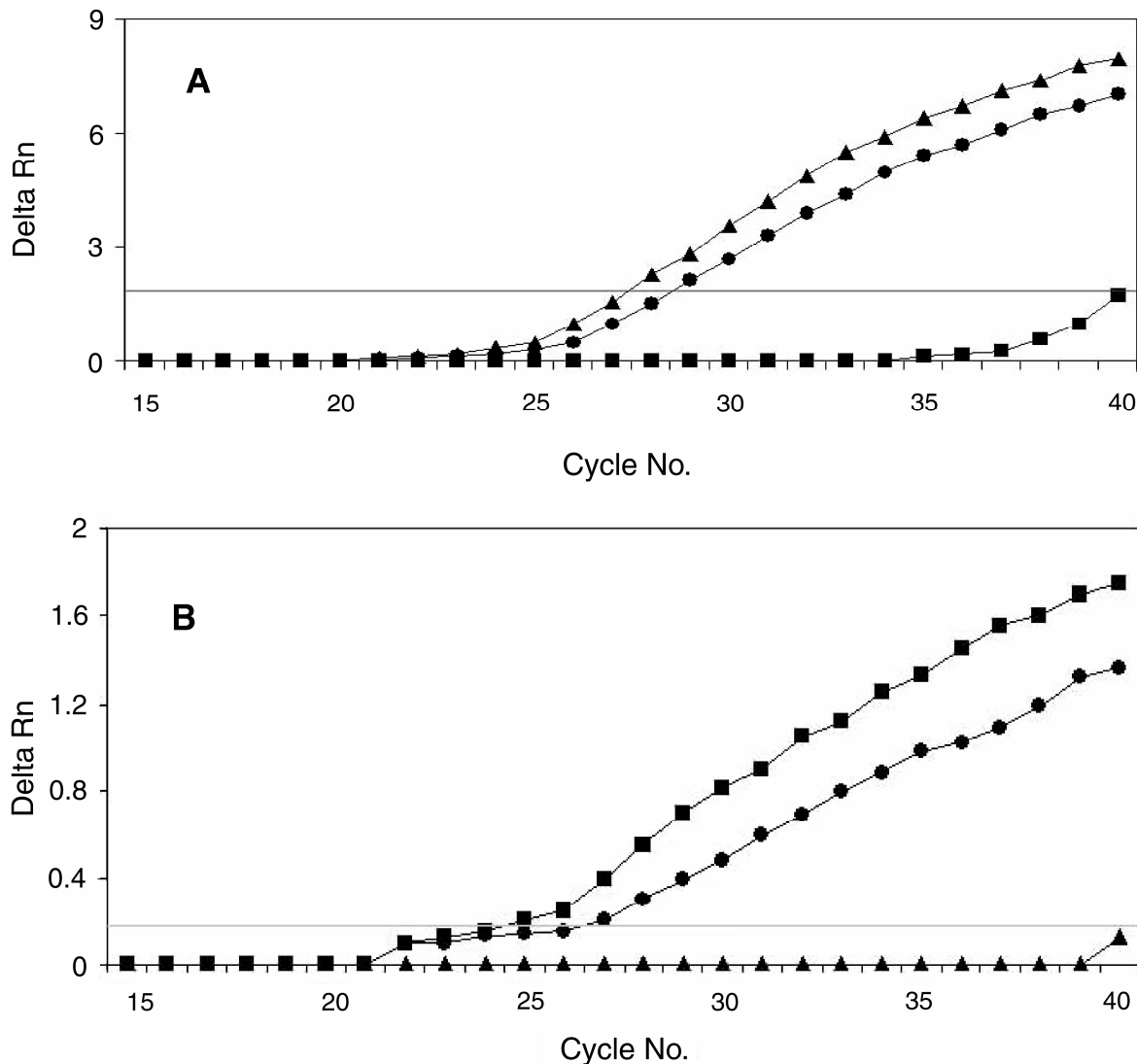


Fig. 1. Real-time amplification plot of total DNA isolated from leaves of individual bean plants having different genotypes for the *bc-l²* and *I* alleles. \blacktriangle = P94207-189A (*bc-l bc-l II*), \bullet = F₁ (*bc-l² bc-l Ii*), and \blacksquare = Olathe (*bc-l² bc-l² ii*). For each reaction, the fluorescent light emission (ΔR_N) is plotted versus the number of polymerase chain reaction cycles. **A**, Amplification plots for the *I*-specific primer/probe set I359PF-I382TC-I436PR (threshold value of $\Delta R_N = 2.0$). **B**, Amplification plots for the *bc-l²*-specific primer/probe set p43335F-p43T369C-p43424R (threshold value of $\Delta R_N = 0.2$).

TABLE 1. Shapiro-Wilk W test for normal distribution, range of means, and group means and standard deviations (SD) for multiplex real-time polymerase chain reaction (PCR) results of F₁ and F₂ bean plants having homozygous or heterozygous genotypes for *bc-l²* and *I*^a

Genotype	N	Shapiro-Wilk W test	Range of means ^b	Group mean \pm SD
<i>Ii</i> (F ₁)	11	W = 0.96, Prob < W = 0.17	40.25 – 54.41	49.80 \pm 4.53
<i>Ii</i> (F ₂)	94	W = 0.99, Prob < W = 0.78	38.24 – 55.25	48.17 \pm 4.20
<i>II</i> (F ₂)	41	W = 0.98, Prob < W = 0.40	76.29 – 107.61	91.91 \pm 7.47
<i>bc-l² bc-l</i> (F ₁)	11	W = 0.97, Prob < W = 0.58	35.66 – 52.98	45.42 \pm 5.46
<i>bc-l² bc-l</i> (F ₂)	104	W = 0.99, Prob < W = 0.99	31.71 – 52.88	43.22 \pm 3.88
<i>bc-l² bc-l²</i> (F ₂)	44	W = 0.98, Prob < W = 0.52	72.50 – 99.56	88.12 \pm 6.68

^a Data were considered to have a normal distribution when (Prob < W) > 0.05. Multiplex real-time PCR reactions contained 100 ng of genomic DNA as template. Plants were genotyped for *I* and *bc-l²* using the primer/probe sets I359PF-I382TC-I436PR and p43335F-p43T369C-p43424R, respectively. F₂ bean plants are the population derived from a cross between P94207-189A (*bc-l bc-l II*) and cv. Olathe (*bc-l² bc-l² ii*).

^b Individual plant means were calculated based on the results of three replicate real-time PCR reactions.

bc-1). Classification of plant genotype based on the results of real-time PCR indicated that the 198 F_2 plants segregated as follows for the *bc-1²* allele: 44 *bc-1² bc-1²*; 104 *bc-1² bc-1*; 50 *bc-1 bc-1*. This did not deviate significantly from the expected 1:2:1 segregation ratio ($\chi^2 = 0.87$; df = 2; $0.30 < P < 0.50$).

Comparison between genotyping both loci with multiplex PCR and F_3 progeny testing. The genotypic segregation ratios for the F_2 population determined by (i) multiplex real-time PCR and (ii) F_3 progeny testing are listed in Table 2. Plants were identified for each of nine possible genotypic classes. A χ^2 analysis indicated that genotypic segregation ratios did not significantly deviate from expected ratios, irrespective of whether genotype was determined based on multiplex real-time PCR or progeny testing (Table 2). Agreement between the two different methods for genotyping was 100% (198/198) for the *bc-1²* allele. Agreement between the two different methods was 92.4% (183/198) for the *I* allele. Differences in genotype assignment for the *I* allele between results based on multiplex real-time PCR and results

based on F_3 progeny testing were most likely due to recombination between the SW13₆₉₀ SCAR marker and the *I* allele, resulting in a recombination distance between SW13₆₉₀ and *I* of 3.87 centimorgans (cM) \pm 0.99.

DISCUSSION

In this report, we describe the development of a multiplex real-time PCR assay that can assign genotypes in a segregating common bean population simultaneously for both the *bc-1²* and *I* alleles. The primer/probe set I359PF-I382TC-I436PR was specifically designed for this study to genotype plants for the *I* allele, whereas the primer/probe set p43335F-p43T369C-p43424R (20) was used to genotype plants for the *bc-1²* allele. The precision of both primer/probe sets used in the multiplex assay is reflected in the relationship between the log₁₀ of the initial DNA template quantity and C_T values observed for standard curve reactions, with $R^2 \geq 0.997$ for both primer/probe sets. The primer/probe set

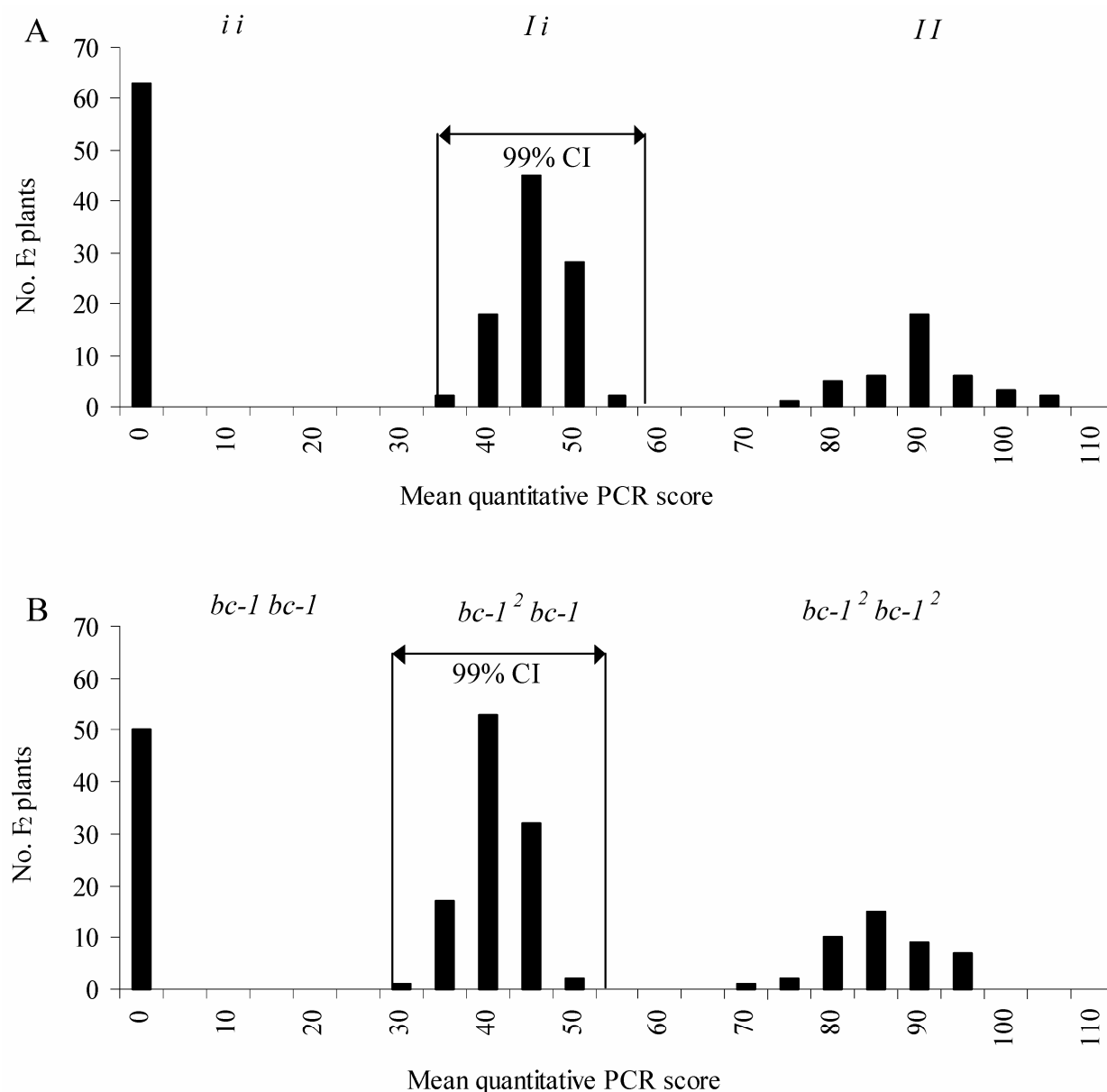


Fig. 2. Frequency histogram for the mean of three multiplex real-time polymerase chain reactions (PCRs) for each of 198 F_2 plants derived from the cross between pinto bean breeding line P94207-189A (*bc-1 bc-1 II*) and cv. Olathe (*bc-1² bc-1² ii*). PCR was performed for each plant using 100 ng of total genomic DNA. The 99% probability distributions for the heterozygous genotypes are presented. The genotype classes of F_2 plants based on their position on the histogram also are presented. **A**, Frequency histogram for the *I* allele (primer/probe set I359PF-I382TC-I436PR). **B**, Frequency histogram for the *bc-1²* allele (primer/probe set p43335F-p43T369C-p43424R).

I359PF-I382TC-I436PR was specific for the SW13₆₉₀ marker linked to the *I* allele, whereas the primer/probe set p43335F-p43T369C-p43424R was specific for the SBD5₁₃₀₀ marker linked to the *bc-l²* allele (Fig. 1).

To perform a multiplex PCR assay, the 5' termini of the TaqMan probes I382TC and p43T369C were labeled with the fluorochromes 6-FAM and VIC, respectively, so that different amplicons specifically produced by each primer/probe set could be detected simultaneously in a single reaction. These two fluorochromes were selected because of the large difference in their respective maximum emission of fluorescence (6-FAM, $\lambda_{\text{max}} = 518$ nm; VIC, $\lambda_{\text{max}} = 554$ nm) (2). The difference between the two fluorochromes in the wavelength of their maximum emission of fluorescence minimizes the spectral overlap between the two fluorochromes, which allows for more accurate quantification of two different amplicons in a single tube (2).

For both primer/probe sets, normal distributions were observed for quantitative PCR results among the reference heterozygous *F*₁ population (Table 1). Normal distributions also were observed among *F*₂ plants classified as heterozygotes, and among *F*₂ plants classified as homozygotes for either the *I* or *bc-l²* alleles (Table 1). Several sources likely contribute to the observed variation, including errors in DNA quantification and differences in the efficiency of template amplification among real-time PCR reactions. For completely efficient PCR reactions performed using samples for which the DNA concentrations were absolutely accurate and precisely quantified, it would be expected that the ratio of PCR results for homozygous plants/PCR results for heterozygous plants would equal 2.0. In this report, for primer/probe set p43335F-p43T369C-p43424R, the ratio of means for homozygous plants/heterozygous plants was $88.12/43.22 = 2.04$. For primer/probe set I359PF-I382TC-I436PR, the ratio of means for homozygous plants/heterozygous plants was $91.91/48.17 = 1.91$. Results with both primer/probe sets fell within 5% of the idealized ratio of 2.0, suggesting that a high degree of precision was maintained throughout the processes of DNA quantification and real-time PCR reactions.

For both the *I* and *bc-l²* alleles, a very high level of agreement was observed between the assignment of plant genotype based on multiplex real-time PCR and assignment based on progeny testing (Table 2). Agreement between the two different methods for genotyping plants for the *bc-l²* allele was 100% (198/198). These results suggest an absence of recombination between SBD5₁₃₀₀, which contains the amplification product, and the linked *bc-l²* allele. For the *I* allele, agreement between the two different methods for genotyping plants was 92.4% (183/198). Differences in genotype assignment between results based on multiplex real-time PCR and results based on *F*₃ progeny testing most likely are due to recombination between the SW13₆₉₀ SCAR marker and the

I allele. Identification of another marker that is more tightly linked to the *I* allele could improve accuracy of *I* allele genotyping.

In this study, a recombination distance of 3.87 ± 0.99 cM was calculated between SW13₆₉₀ and the *I* allele. These results are similar to previously reported recombination distances between SW13₆₉₀ and the *I* allele, which have ranged from 1.0 cM (14) to 8.9 cM (3). This resulted in three plants of the desired genotype (*bc-l² bc-l² I I*) being erroneously genotyped as heterozygous for *I* (*bc-l² bc-l² I i*) based on the results of real-time PCR. These plants would have been excluded as a result of this erroneous genotyping. Of greater consequence, recombination between the SW13₆₉₀ SCAR marker and the *I* allele resulted in a single plant being erroneously genotyped as homozygous for *I* (*bc-l² bc-l² I I*), when results of *F*₃ progeny testing indicated that the plant was heterozygous for *I* (*bc-l² bc-l² I i*). This plant would have been selected based on genotyping with real-time PCR despite having a single copy of the deleterious *i* allele. However, incorrect assignment of genotype should not be considered to be due to limitations inherent in this approach of using real-time PCR to allow for co-dominant interpretation of a dominant marker, but rather as a consequence of recombination between SW13₆₉₀ and the *I* allele. Erroneous genotyping due to recombination between a marker and a locus of interest also can occur using inherently co-dominant markers, such as simple sequence repeats (1), restriction fragment length polymorphisms (4), and cleaved amplified polymorphic sequences (13).

For the most efficient selection of plants having the desired homozygous genotype (*bc-l² bc-l² I I*), breeders could use a combinatorial approach that initially used the multiplex real-time PCR assay to identify homozygous plants. The *F*₂ plants that the multiplex real-time PCR assay indicated had the homozygous genotype then could be subjected to *F*₃ progeny testing to confirm genotype. Using this approach, in this study, only the four plants that the multiplex real-time PCR assay indicated had the *bc-l² bc-l² I I* genotype (Table 2) would be subsequently examined by *F*₃ progeny testing. This combinatorial approach would have required only four *F*₃ progeny tests to identify three *F*₂ plants with the desired *bc-l² bc-l² I I* genotype. In contrast, 104 *F*₃ progeny tests would be necessary to identify six *F*₂ plants with the desired genotype, if selection of *F*₂ plants was based solely on the use of the dominant markers SW13₆₉₀ and SBD5₁₃₀₀ to identify plants with at least a single dominant allele at both loci (*bc-l² _ I _*) (Table 2).

Substantially reducing the number of *F*₃ family progeny tests would considerably reduce costs for breeding programs. This would be especially true for progeny tests that evaluate disease reaction, which occasionally fail completely due to changes in virulence of pathogen isolates or an inability to maintain greenhouse conditions that are conducive to disease development and symptom expression. Additionally, it should be possible to completely eliminate progeny testing if repeated linkage analysis indicated an absence of recombination between the loci of interest and the amplicon produced by the real-time PCR assay. The ability to identify desired genotypes at the seedling stage would greatly accelerate progress in germ plasm enhancement and cultivar development.

TABLE 2. A χ^2 analysis of genotypic segregation ratios determined by multiplex real-time polymerase chain reaction (PCR) and *F*₃ progeny testing for an *F*₂ population derived from the cross P94207-189A (*bc-l bc-l I I*) \times Olathe (*bc-l² bc-l² i i*)

Genotype	Expected (<i>N</i> = 198)	Observed	
		Multiplex real-time PCR ^a	<i>F</i> ₃ progeny testing ^b
<i>bc-l² bc-l² I I</i>	12.375	4	6
<i>bc-l² bc-l² I I</i>	24.75	24	22
<i>bc-l² bc-l² i i</i>	12.375	16	16
<i>bc-l² bc-l I I</i>	24.75	25	26
<i>bc-l² bc-l I I</i>	49.5	51	51
<i>bc-l² bc-l i i</i>	24.75	28	27
<i>bc-l bc-l I I</i>	12.375	12	13
<i>bc-l bc-l I I</i>	24.75	19	22
<i>bc-l bc-l i i</i>	12.375	19	15

^a $\chi^2 = 12.12$ (df = 8) $0.1 > P > 0.2$.

^b $\chi^2 = 5.86$ (df = 8) $0.5 > P > 0.7$.

LITERATURE CITED

1. Akkaya, M. S., Bhagwat, A. A., and Cregan, P. B. 1992. Length polymorphisms of simple sequence repeat DNA in soybean. *Genetics* 132: 131-139.
2. Applied Biosystems. 2001. ABI PRISM 7700 Sequence Detection System User Bulletin #5: Multiplex PCR with TaqMan VIC probes. Applied Biosystems. Foster City, CA.
3. Ariyaratne, H. M., Coyne, D. P., Jung, G., Skroch, P. W., Vidaver, A. K., Steadman, J. R., Miklas, P. N., and Bassett, M. J. 1999. Molecular mapping of disease resistance genes for halo blight, common bacterial blight, and bean common mosaic virus in a segregating population of common bean. *J. Am. Soc. Hortic. Sci.* 124:654-662.
4. Beckman, J. S., and Soller, M. 1983. Restriction fragment length poly-

- morphisms in genetic improvement: Methodologies, mapping and costs. *Theor. Appl. Genet.* 67:35-43.
5. Collmer, C. W., Marston, M. F., Taylor, J. C., and Jahn, M. 2000. The *I* gene in bean: A dosage-dependent allele conferring extreme resistance, hypersensitive resistance, or spreading vascular necrosis in response to the Potyvirus *Bean common mosaic virus*. *Mol. Plant-Microbe Interact.* 13:1266-1270.
 6. Drijfhout, E. 1978. Genetic interaction between *Phaseolus vulgaris* and bean common mosaic virus resistance with implications for strain identification and breeding for resistance. Centre Agric. Publ. Doc., Wageningen, The Netherlands.
 7. Fehr, W. R. 1987. Principles of Cultivar Development. Volume 1: Theory and Technique. Macmillan Publishing, NY.
 8. Fisher, M. L., and Kyle, M. M. 1994. Inheritance of resistance to potyviruses in *Phaseolus vulgaris* L. III. Cosegregation of phenotypically dominant responses to nine potyviruses. *Theor. Appl. Genet.* 89:818-823.
 9. Haley, S. D., Afanador, L., and Kelly, J. D. 1994. Identification and application of a random amplified polymorphic DNA marker for the *I* gene (Potyvirus resistance) in common bean. *Phytopathology* 84:157-160.
 10. Heid, C. A., Stevens, J., Livak, K. J., and Williams, P. M. 1996. Real time quantitative PCR. *Genome Res.* 6:986-994.
 11. Holland, P. M., Abramson, R. D., Watson, R., and Gelfand, D. 1991. Detection of specific polymerase chain reaction products by utilizing the 5'-3' exonuclease activity of *Thermus aquaticus* DNA polymerase. *Proc. Natl. Acad. Sci. USA* 88:7276-7280.
 12. Kelly, J. D., Afanador, L., and Haley, S. D. 1995. Pyramiding genes for resistance to bean common mosaic virus. *Euphytica* 82:207-212.
 13. Konieczny, A., and Ausubel, F. M. 1993. A procedure for mapping *Arabidopsis* mutations using co-dominant ecotype-specific PCR-based markers. *Plant J.* 4:403-410.
 14. Melotto, M., Afanador, L., and Kelly, J. D. 1996. Development of a SCAR marker linked to the *I* gene in common bean. *Genome* 39:1216-1219.
 15. Miklas, P. N., Larsen, R. C., Riley, R., and Kelly, J. D. 2000. Potential marker-assisted selection for *bc-1²* resistance to bean common mosaic potyvirus in common bean. *Euphytica* 116:211-219.
 16. Nasarabadi, S., Milanovich, F., Richards, J. F., and Belgrader, P. 1999. Simultaneous detection of Taqman probes containing Fam and Tamra reporter fluorophores. *Biotechniques* 27:1116-1117.
 17. Ott, R. L. 1993. An Introduction to Statistical Methods and Data Analysis. Wadsworth, Inc., Belmont, CA.
 18. Paran, I., and Michelmore, R. W. 1993. Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. *Theor. Appl. Genet.* 85:985-993.
 19. Shapiro, S. S., and Wilk, M. B. 1965. An analysis of variance test for normality (complete samples). *Biometrika* 52:591-611.
 20. Vandemark, G. J., and Miklas, P. N. 2002. A fluorescent PCR assay for the codominant interpretation of a dominant SCAR marker linked to the virus resistance allele *bc-1²* in common bean. *Mol. Breed.* 10:193-201.
 21. Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A., and Tingey, S. V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids. Res.* 18:6531-6535.
 22. Wood, D. R., and Keenan, J. G. 1982. Registration of 'Olathe' bean. *Crop Sci.* 22:1259-1260.